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The Chemistry of Xanthine Oxidase

11. ULTRACENTRIFUGE AND GEL-FILTRATION STUDIES ON THE MILK ENZYME*

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The molecular weight of xanthine oxidase from cow's milk, as measured by sedimentation and diffusion, has been given as 290 000 (Avis, Bergel, Bray, James & Shooter, 1956*a*). The value from chemical analysis, on the basis of 2 moles of FAD/mole, namely 308 000, was in agreement with this (Avis, Bergel & Bray, 1956*b*). Recently Brumby (1963) and Brumby & Massey (1963) purified pig-liver xanthine oxidase and showed it to be generally similar to the milk enzyme. However, though Brumby (1963) found a molecular weight of 288 000 by the sedimentation and diffusion method, Brumby & Massey (1963) (see also Brumby, 1963) give values of 385 000 obtained by the Archibald method and 190 000 by gel filtration, suggesting that reversible dissociation of a dimer might be occurring. In the present paper we report further studies on the molecular weight of the milk enzyme which confirm our original value, and we show also that the molecule is broken down only under relatively drastic conditions.

MATERIALS AND METHODS

Xanthine oxidase solutions. The enzyme was prepared from milk as described previously (Palmer, Bray & Beinert, 1964; cf. Gilbert & Bergel, 1964). A single batch of twice-chromatographed xanthine oxidase (sample A) was used for all the experiments, apart from one gel-filtration run, for which cruder material (sample B) was used. This sample had not been chromatographed and had lost activity during storage. At the time of use sample A had an activity/E_{450} of about 80 (cf. Bray, Pettersson & Ehrenberg, 1961), whereas sample B had a value of only 35. Xanthine oxidase concentrations were determined either directly from E_{450} (Avis *et al.* 1956*b*) or indirectly, in some ultracentrifuge runs, by comparison of the areas under the peaks with those given by samples of known concentration. All solutions used in the ultracentrifuge work contained sodium salicylate (1 mM) and EDTA (1 mM) to stabilize the enzyme (Bergel & Bray, 1959).

Proteins for gel filtration. These are listed in Table 3. Apoferritin and lactoperoxidase were kindly provided by Dr Pauline M. Harrison and Mr A. Pickering respectively.

Sedimentation experiments. These experiments were performed in a Spinco model E analytical ultracentrifuge. The temperature was controlled near to 20° unless otherwise stated. For the determinations of molecular weight solutions of xanthine oxidase in 0·2 M-NaCl buffered to pH 6·0 with 0·01 M-phosphate were used. At the highest enzyme concentration (0·89%) about 2% of a contaminant with a sedimentation coefficient of about 16–17 S was observed. This was presumably the component *fa* described by Avis *et al.* (1956*a*) and by Palmer *et al.* (1964). The presence of this small amount of impurity will not affect the calculations of molecular weight reported below. For the measurements of the diffusion coefficient a 0·89% solution was used. Solvent against which the enzyme had been dialysed was layered on top of the solution in a synthetic-boundary cell. Photographs were taken every 8 min. for 80 min. with the rotor spinning at 7852 rev./min. The schlieren diagrams were projected on to squared paper and tracings were made of the inside and outside regions of the curves by two different operators. The diffusion coefficient was calculated from the regression of $(A/H)^2$ on t, by using the formula:

$$(A/H)^2 = 4\pi Dt$$

* Part 10: Gilbert (1964).

where A is the area under the curve, H the height of the curve, D the diffusion coefficient and t the time.

The same solution of enzyme was used for determinations of the molecular weight by the Archibald method. The rotor was spun at 7852 rev./min. and photographs were taken every 8 min. for 124 min. Calculations were performed as described by Klainer & Kegeles (1955). Measurements at the air–solution meniscus only were used, since the refractive-index gradient at the cell bottom was too steep for reliable extrapolation. Values of the molecular weight were calculated from each photograph.

For experiments with sodium thioglycollate, the solid (Difco, Detroit, Mich., U.S.A.) was dissolved in 8 M-urea and the solution (0·1 M) was used immediately to dilute the enzyme for the run.

Preparation and calibration of Sephadex G-200 column. Investigation of the properties of Sephadex G-200 gel-filtration medium has shown that the swelling of the gel in buffer solution continues for several months, resulting in a rise in the upper exclusion limit of the gel and concurrently some loss in resolving power for small protein molecules (P. Andrews, unpublished work). The experiments described below were performed with Sephadex G-200 (lot no. To 3016; particle size 200–300 mesh) (Pharmacia, Uppsala, Sweden) that had been soaked in 0·05 M-tris hydrochloride buffer, pH 7·5, containing KCl (0·1 M), for about 3 months. A column of the swollen gel was packed in the cold by the method recommended by Flodin (1961), and equilibrated with the buffered KCl solution used for swelling the gel. Samples were diluted to 2 ml. with the same medium for application to the top of the column. Effluent was collected in 3 ml. portions by using a fraction collector (Aimer Products Ltd., London) fitted with a siphon. All column runs were carried out at 0–5°.

The proteins of known molecular weights listed in Table 3 were used in various combinations, usually three at a time, in experiments to determine their elution volumes from the column, and thus to calibrate the column for the determination of molecular weight. The quantities used (0·5–3 mg.) were such as to permit the estimation of protein in the column effluents by spectrophotometry at 220 or 230 mμ, and in addition aldolase activity was estimated by the coupled assay with excess of glyceraldehyde 3-phosphate dehydrogenase (Taylor, 1955). The void volume of the column (elution volume of material excluded from the gel pores) was determined in an experiment with indian ink. Elution volumes and the void volume were estimated to the nearest 1 ml. from elution diagrams. More information on the preparation and operation of Sephadex columns for the determination of molecular weights has been given by Andrews (1964).

Gel-filtration experiments. For the determination of the molecular weight, xanthine oxidase (sample A) (0·3 and 1·0 mg. in separate runs) was subjected to gel filtration on the Sephadex G-200 column in admixture with aldolase (1·5 mg.) and bovine serum albumin (2·5 mg.), whereas no other proteins were added to either xanthine oxidase (sample A) (1·0 and 3·5 mg.) or xanthine oxidase (sample B) (10 mg.) in experiments designed to study their heterogeneity. Protein in column effluents was estimated as indicated above and xanthine-oxidase activity by measuring the rate of extinction change at 295 mμ with 0·1 mM-xanthine in 0·13 M-tris hydrochloride buffer, pH 8·0, at 25° (cf. Avis, Bergel & Bray, 1955).

RESULTS

Molecular weight from sedimentation experiments. The molecular weight of xanthine oxidase has been determined from the sedimentation and diffusion coefficients and also by the approach-to-equilibrium method.

Fig. 1 shows that the sedimentation coefficient exhibits little concentration-dependence in the range from 0·89 to 0·02 %. Calculation of the intercept at zero concentration from the regression of S on concentration gave $S^0_{20,w}$ 11·30 ± 0·05s, the error being taken as the standard error of the extrapolated regression line at the ordinate (Ezekiel & Fox, 1961). These results are in agreement with previously reported values (S^0 11·4s; Avis *et al.* 1956*a*) and extend them by providing more data at low concentrations of xanthine oxidase.

Data on the diffusion coefficient are summarized in Table 1. The mean value is $D_{20,w}$ (3·93 ± 0·30) × 10^{-7} cm.^{2} sec.$^{-1}$. The result is in agreement with that reported by Avis *et al.* (1956*a*).

The partial specific volume, $\bar{v}$, of xanthine oxidase, determined from density measurements, was given as 0·74 ± 0·02 by Avis *et al.* (1956*a*). This constant may also be calculated (Schachman, 1957) from the amino acid composition, which is now available (Bray & Malmström, 1964). The value

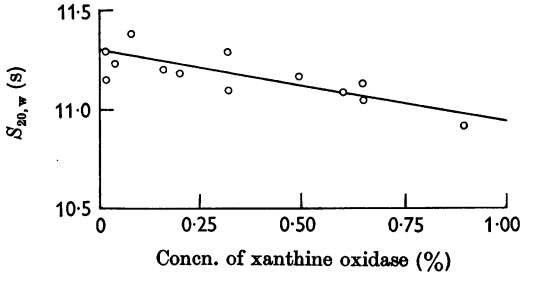

Fig. 1. Concentration-dependence of the sedimentation coefficient of xanthine oxidase. Experiments were carried out in 0·2 M-NaCl, pH 6·0. The line is the regression of S on concentration.

Table 1. *Diffusion coefficient of xanthine oxidase*

Measurements were carried out in the ultracentrifuge with a xanthine oxidase concn. of 0·89 %. The error was obtained from the standard error of the regression coefficients of $(A/H)^2$ on t. Details are given in the text.

	$10^7 D_{20,w}$ (cm.^{2} sec.$^{-1}$)	
	Operator 1	Operator 2
Outside	3·91	4·06
Inside	3·95	3·79
Mean (±S.E.M.)	3·93 (±0·30)	

thus obtained, 0·737, is in excellent agreement with the earlier value. Since limits of error cannot readily be fixed for the value calculated from the analysis, the earlier value is used in the calculations below.

Combination of the data for sedimentation, diffusion and partial specific volume gives a value of 274 000 for the molecular weight, M. Combination of the errors on S^0 and D by taking the root of the sum of their squares gives the error on M as $\pm 21\,000$. If errors on $\bar{v}$ are included the error becomes $\pm 30\,000$. Results from the Archibald method are given in Table 2. The mean of the four sets of 12 observations is 265 000, and the error (S.E.M.) is ± 9000. If errors on $\bar{v}$ are included the value becomes $265\,000 \pm 22\,000$.

Molecular weight from gel filtration. Experiments with different concentrations of xanthine oxidase (sample A), either alone or admixed with 'standard' proteins, gave essentially the same value (131 ± 1 ml.) for the elution volume, V_e of the main component. This was interpreted in terms of molecular weight with the calibration curve for the column given in Fig. 2, and corresponds to a value of $286\,000 \pm 35\,000$. This value is the one obtained from the regression of log M on V_e, with the error taken as the root of the square of the standard error of the regression line at $V_e = 131$ ml. plus the square of the error arising from the uncertainty of V_e.

Minor components detected by sedimentation. Apart from minor contamination with *fa*, which could be detected only at the highest concentration used, the schlieren diagrams showed only one peak at all concentrations at pH 6.

Minor components detected by gel filtration. Gel filtration on Sephadex G-200 indicated that xanthine oxidase (sample A) consisted mainly of one component, which accounted for about 92 % of the protein of the preparation (based on extinction at 230 mμ) and possessed over 99 % of its enzymic activity, but other components were present which also had xanthine-oxidase activity (Fig. 3). Homogeneity of the enzyme forming the larger part of the main peak in the elution diagram was indicated by constant specific activity (xanthine-oxidase activity related to extinction at 230 mμ) in fractions covering the elution volume range 120–153 ml. The recovery of enzymic activity was quantitative, calculated relative to the activity of enzyme dissolved in column eluent and kept in the cold for the duration of a column run (16 hr.).

The minor peak in the elution diagram (Fig. 3) represents material that emerged from the column at the void volume (82 ml.). At the peak, its specific activity was 7 % of that of the enzyme forming the bulk of the preparation.

Table 2. *Molecular weight of milk xanthine oxidase by the Archibald method*

Details are given in the text.

	$10^{-5} \times$ Molecular weight	
	Operator 1	Operator 2
Outside	2·60 ± 0·15	2·44 ± 0·40
Inside	2·65 ± 0·22	2·84 ± 0·53
Mean (± S.E.M.; total of 12 determinations)	2·65 ± 0·09	

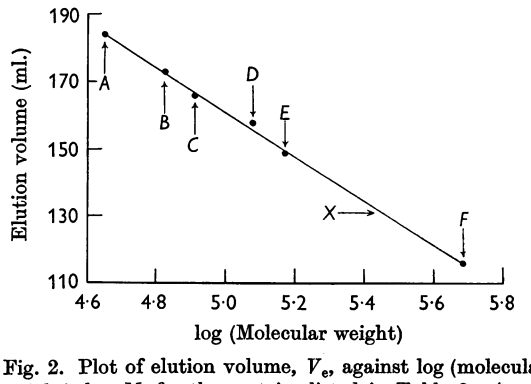

Fig. 2. Plot of elution volume, V_e, against log (molecular weight), log M, for the proteins listed in Table 3: *A*, ovalbumin; *B*, serum albumin; *C*, lactoperoxidase; *D*, glyceraldehyde 3-phosphate dehydrogenase; *E*, aldolase; *F*, apoferritin; *X* indicates the elution volume of xanthine oxidase. The line is the regression of log M on V_e.

Table 3. *Proteins used in standardizing the Sephadex G-200 gel-filtration column for the determination of molecular weights*

Protein	Description (supplier)	Molecular weight	Reference
Ovalbumin	2 × Crystallized (L. Light and Co. Ltd.)	45 000	Warner (1954)
Serum albumin (bovine)	Fraction V (Sigma Chemical Co.)	67 000	Phelps & Putnam (1960)
Lactoperoxidase	Purified by method of Morrison, Hamilton & Stotz (1957)	82 000	Polis & Shmukler (1953)
Glyceraldehyde 3-phosphate dehydrogenase (from rabbit muscle)	Crystallized (Boehringer und Soehne G.m.b.H.)	120 000	Taylor & Lowry (1956)
Aldolase (from rabbit muscle)	Crystallized (Boehringer und Soehne G.m.b.H.)	149 000	Taylor & Lowry (1956)
Apoferritin	2 × Crystallized	480 000	Harrison (1963)

Crude xanthine oxidase (sample B) was examined for the presence of high-molecular-weight material with xanthine-oxidase activity. None was detected, although the amount of material absorbing at 230 mμ emerging from the column at the void volume, relative to the enzymic activity present in the sample, was seven times that in sample A.

Effect of solvent and pH *on sedimentation behaviour.* At pH 6 no evidence for any decrease in sedimentation coefficient at low concentrations of xanthine oxidase has been observed (Fig. 1), nor, in the measurement of the molecular weight by the Archibald method, was there any drift to lower values at longer sedimentation times. Thus there was no evidence for dissociation of the enzyme under these conditions. Sedimentation coefficients were then measured under a variety of conditions to see if dissociation of the enzyme could be obtained. The results of experiments at different pH values are given in Table 4. Over the range pH 3·5–9·8 only one component was observed. However, at pH 11·7 (0·02N-sodium hydroxide) and higher, components with lower sedimentation coefficients were observed in addition to xanthine oxidase. In the experiment illustrated (Fig. 4) the component with S 3·7s accounted for 56 % of the total protein. A number of further experiments were performed at high pH values. In one at pH 11·8 the enzyme was about 50 % degraded to a component with S 1·95s. After neutralization this component was still present, but the area under the peak had decreased significantly. In another experiment in the cold at pH 11·8 three peaks including one for xanthine oxidase were observed, the two slower ones having sedimentation coefficients of 0·47 and 7·75s. In all these experiments there was some loss of activity but little change in the absorption spectrum. There were no indications that the degradation products were coloured. Small increases in the sodium hydroxide concentration were accompanied by much more extensive degradation of the enzyme. Both at pH 12·1 (0·04N-sodium hydroxide) and at pH 12·6 (0·2N-sodium hydroxide) almost complete loss of activity and a change in the colour of the solution from brown to yellow took place. At pH 12·1, after neutralization and removal of a precipitate, the ultracentrifuge showed only slowly sedimenting components (S 0·5s). From these observations it seems unlikely that even in the experiment in Fig. 4 the enzyme is dissociating into simple sub-units.

An experiment was also performed at pH 1·9 (0·02N-hydrochloric acid). The solution became yellow at once and the ultracentrifuge showed that

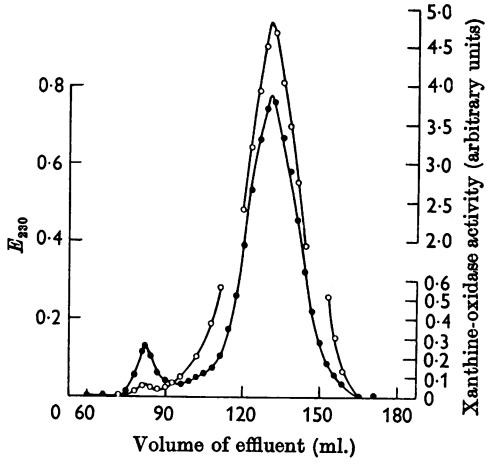

Fig. 3. Elution diagram of 3·5 mg. of xanthine oxidase (sample A) from a Sephadex G-200 column (53 cm. × 2·3 cm. diam.). ○, E_{230}; ●, xanthine-oxidase activity. Experimental details are given in the text.

Table 4. *Effect of* pH *on sedimentation of xanthine oxidase*

Details are given in the text. The xanthine-oxidase concn. was 0·73 %.

pH	Buffer	$S_{20,w}$ (s)
3·5*	Acetate (0·1M)–NaCl (0·1M)	10·6
4·7	Acetate (0·1M)–NaCl (0·1M)	10·4
6·0	Phosphate (0·01M)–NaCl (0·2M)	10·9
9·8	Carbonate (0·1M)–NaCl (0·1M)	10·5
11·8*†	NaOH (0·02N)–NaCl (0·05M)	10·1‡

* Partial loss of activity under these conditions.
† See also Fig. 4 and text.
‡ Mean of four experiments.

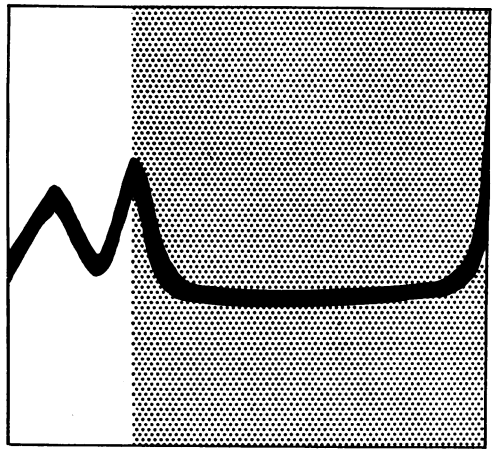

Fig. 4. Sedimentation diagram for xanthine oxidase at pH 11·7. In a control experiment at a lower pH only the peak on the right, representing the undegraded enzyme, could be detected.

complete breakdown to a component with S 4·0s had taken place. The peak was asymmetric and colour was associated with it.

The sedimentation coefficient of xanthine oxidase itself (Table 4) is at a maximum near the isoelectric point, pH 5·3–5·4 (Avis *et al.* 1956*a*), and decreases slightly at higher and lower pH values. It appears that changes of pH in the range studied lead only to comparatively small changes in the shape or degree of hydration of the enzyme. Above pH 6 the shape changes progressively until at pH 11·7, which seems to be a critical point, breakdown of the enzyme to smaller units also occurs.

Three further media that might dissociate the enzyme have been tried. Morell (1952) noted that calcium chloride splits FAD from the enzyme. An experiment was therefore carried out in the presence of 0·8M-calcium chloride in 0·01M-tris–maleate buffer, final pH 4·3, at about 10°. As expected, the solution became somewhat yellow. The sedimentation coefficient, $S_{20,w}$, was 9·0s (concn.: 0·6 %); only one peak was observed. Sedimentation coefficients in 6·7M-urea with and without thioglycollate (cf. Lindley, 1955) were respectively 9·8 and 8·3s (concn.: 0·6 %). However, because of the large viscosity corrections that had to be applied, these are not regarded as particularly reliable values.

DISCUSSION

Molecular weight. The present data from sedimentation and diffusion, from the Archibald method and from gel filtration are in good agreement and may be combined to give the molecular weight of milk xanthine oxidase as 275 000, a value that agrees well with the earlier result of Avis *et al.* (1956*a*).

The standard proteins employed for gel filtration form a consistent series, since the elution volume is accurately proportional to the logarithm of the molecular weight (Fig. 2). There is, however, no absolute certainty that xanthine oxidase fits into this series with regard to the correlation between behaviour on Sephadex and molecular weight. Nevertheless, the molecular weight obtained by gel filtration is regarded as valuable confirmation of the ultracentrifuge data.

The molecular weight (288 000) of xanthine oxidase isolated from pig liver determined from sedimentation velocity and diffusion studies (Brumby, 1963) agrees well with the value reported above. However, gel-filtration experiments gave a molecular weight of 190 000 and the approach-to-equilibrium method a value of 385 000 (Brumby & Massey, 1963). From this it might be concluded that the pig-liver enzyme is a dimer that dissociates at low concentration. No evidence has been found to suggest that the enzyme from cow's milk dissociates at low concentration. Though there is no *a priori* reason to assume that the enzyme from the two sources should possess similar physical properties, there are notable similarities between their chemical compositions and enzymic properties and their sedimentation and diffusion behaviour. However, it is somewhat surprising for the pig-liver enzyme, which apparently exhibits concentration-dependent dissociation, that, in the measurement of molecular weight by the approach-to-equilibrium method, no apparent dependence of the molecular weight on sedimentation time seems to have been observed. In gel filtration, even with the range of standard proteins used in the present work, the error in the estimated molecular weight is ± 12 %. The use of fewer standards could easily lead to much greater errors.

The effect of changing pH on the sedimentation characteristics of the enzyme from cow's milk indicates that progressive changes in the shape or hydration of the molecule occur on either side of the isoelectric point. At pH 11·7 and above components of lower sedimentation rate are produced. The variations observed between experiments suggest that these new components are random degradation products rather than simple sub-units of the molecule. The one experiment at pH 1·9, on the other hand, indicated complete breakdown to a component containing iron or flavin or both. Further experiments indicated that the xanthine oxidase molecule is not built from sub-units held together by hydrogen bonds or disulphide bridges.

Nature of impurities. Some contamination of the purified milk xanthine oxidase by higher-molecular-weight impurities was observed both in the ultracentrifuge (see above) and by gel filtration. A lower limit for the molecular weight of the enzymically active contaminant of low specific activity which was eluted from the gel-filtration column at the void volume can be estimated if it is assumed that this material is similar to the 'standard' proteins in its gel-filtration behaviour. Extrapolation of the line in Fig. 2 to the void volume indicates that proteins with molecular weights greater than 1 600 000 would be excluded from the gel pores. However, the probability is that the graph will curve towards higher molecular weights (compare results for other Sephadex gels; Andrews, 1964), and so a minimum molecular weight of perhaps 2 500 000 is indicated. A corresponding peak of very rapidly sedimenting material was not observed in the ultracentrifuge, which strongly suggests that this excluded material is grossly heterogeneous. Two explanations for its presence may be considered. One possibility is that it consisted of enzyme in its true native state associated with lipoprotein, for xanthine oxidase in fresh milk is

associated with lipoprotein complexes attached to the fat globules (Bailie & Morton, 1958; Robert & Polonovski, 1955). However, the fact that similar material was not observed in a less highly purified preparation (sample B) argues against this explanation. Another possibility is that the material consisted of highly aggregated and largely inactivated forms of xanthine oxidase, formed either during the purification procedure or during storage. In this case the presence in the preparation of lower aggregates could also be expected, and in fact the appearance of the elution diagram (Fig. 3) in the region between the two peaks indicates that this might have been so. It has been suggested (Palmer *et al.* 1964) that the contaminant *fa* often seen in the ultracentrifuge, with a sedimentation coefficient of 16–17 s, might be a dimer of xanthine oxidase. The gel-filtration work provides no further information on this, though slight contamination with a dimer, which would be expected to have an elution volume of 111 ml., would be possible according to Fig. 3.

Gel filtration evidently provides a means both of purifying xanthine oxidase from high-molecular-weight contaminants and of isolating these as a preliminary to their further study.

SUMMARY

1. The molecular weight of xanthine oxidase from cow's milk has been determined as 274 000 ± 30 000 from sedimentation and diffusion measurements, 265 000 ± 22 000 by the Archibald method and 286 000 ± 35 000 by gel filtration.

2. Between pH 3·5 and 9·8 there were no indications of dissociation into sub-units. At pH 1·9 and also at pH 11·7 and above, breakdown of the enzyme into smaller units was observed. Dissociation was not brought about by calcium chloride, urea, or urea plus thioglycollate.

3. The nature of contaminants present in small amounts in some preparations and detected in the ultracentrifuge or by gel filtration is discussed briefly.

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